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Use of less-than-lifetime (LTL) durational limits for nitrosamines: Case study of *N*-Nitrosodiethylamine (NDEA)

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ABSTRACT

The ICH M7(R1) guideline describes a framework to assess the carcinogenic risk of mutagenic and carcinogenic pharmaceutical impurities following less-than-lifetime (LTL) exposures. This LTL framework is important as many pharmaceuticals are not administered for a patient's lifetime and as clinical trials typically involve LTL exposures. While there has been regulatory caution about applying LTL concepts to cohort of concern (COC) impurities such as *N*-nitrosamines, ICH M7 does not preclude this and indeed literature data suggests that the LTL framework will be protective of patient safety for *N*-nitrosamines. The goal was to investigate if applying the LTL framework in ICH M7 would control exposure to an acceptable excess cancer risk in humans. Using *N*-nitrosodiethylamine as a case study, empirical data correlating exposure duration (as a percentage of lifespan) and cancer incidence in rodent bioassays indicate that the LTL acceptable intake (AI) as derived using the ICH M7 framework would not exceed a negligible additional risk of cancer. Therefore, controlling *N*-nitrosamines to an LTL AI based on the ICH M7 framework is thus demonstrated to be protective for potential carcinogenic risk to patients over the exposure durations typical of clinical trials and many prescribed medicines.

1. Introduction

In 2008, the International Life Sciences Institute (ILSI) and Health and Environmental Sciences Institute (HESI) held a workshop to develop a framework for less-than-lifetime (LTL) exposures to carcinogens (Felter et al., 2011). The committee was referred to as MISTEC (Methods for Intermittent and Short-Term Exposure to Carcinogens). Members of the committee had a wide representation of scientists including industry, government, and academia. The MISTEC group used information from the literature and regulatory applications to build a risk framework following LTL exposures to carcinogenic substances.

A time and dose relationship in toxicology was first developed in the 1920s; it is known as Haber's law which defines C x T = k, where C is concentration, T is time, and k a constant (Haber, 1924). A practice of using LTL exposure for carcinogens has existed in regulatory guidance since the mid-1980s. In 1986, USEPA guidance stated that it can be assumed that a high dose of a carcinogen received over an LTL scenario is equivalent to a corresponding low dose spread over a lifetime when the total exposure is equivalent (i.e., $k = C_1 \times T_1 = C_2 \times T_2$). Strict Haber's law assumes that there is a linear relationship between time and toxicity. However, there has been concern that over the short-term duration, the risk can be underestimated. For example, for the

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extremely short-duration exposures, additional measures may be needed to protect for potential dose-rate effects (USEPA, 1986). An additional risk-assessment framework for LTL exposures to genotoxic carcinogens was therefore developed, which includes an additional dose-rate correction factor (DCRF) of 10 for extremely short durations (1–10 days) to protect for sensitive subpopulations (Bos et al., 2004).

The framework developed by MISTEC was employed to develop LTL cancer-risk guidance for pharmaceutical mutagenic impurities, otherwise known as ICH M7 in 2014, and further updated in 2017 (ICH, 2017). Many drug-substance-exposure scenarios are LTL. These include medications indicated for a short duration (e.g., antibiotics, topical steroids, etc.) or drug candidates in clinical trials. In ICH M7(R1), there are five different classes of potentially mutagenic impurities (Table 1). The threshold of toxicological concern (TTC) was developed as a highly conservative chronic acceptable intake (AI) for mutagenic impurities (Class 2 and 3 impurities) in pharmaceuticals where carcinogenic potency is unknown (Muller et al., 2006). The lifetime TTC of 1.5 μg/day was based on a large database of carcinogens and is considered the dose with a high probability of not exceeding a 1 in 100,000 excess cancer risk. Also included was an LTL framework for mutagenic pharmaceutical impurities, which initially was referred to as the "staged"-TTC. As part of the ICH M7 guidance, the LTL concept was developed for mutagenic impurities based on different patient-exposure durations (Table 2).

ICH M7 also describes a process for developing compound-specific limits for mutagenic carcinogens (Class 1 impurities). The primary method, assuming a no-threshold mechanism, is performing linear extrapolation from a TD_{50} (dose that results in a 50% excess tumor incidence). A no-threshold assumption implies that a carcinogenic response can occur at any dose. Other methods can be used for deriving carcinogenic potency, such as benchmark dose (dose that result in percent response over background (e.g., 10%) for quantal data) can provide some advantages over the TD_{50} such as modeling the lower end of the dose-response curve (EFSA, 2017; USEPA, 2012). Nonetheless, TD_{50} has been the primary cancer potency estimate used for the derivation of the AI for *N*-nitrosamine impurities (EMA, 2020a). Although linear extrapolation from TD_{50} implies there is no threshold, for many mutagenic carcinogens a threshold dose has been demonstrated based mainly on the fact that there is a dose below which DNA-repair mechanisms are able to prevent carcinogenic outcomes (Clewell et al., 2019; Johnson et al., 2014; Kobets and Williams, 2019; MacGregor et al., 2015; Waddell, 2004).

The LTL AIs can also be applied to compound-specific limits, based on the same multiples of the lifetime TTC, as shown in Table 2 for illustration. As described in Note 6 of ICH M7(R1), LTL limits do not assume strict linearity between dose, time and response (i.e., C x T = k).

Table 1
Classification of impurities and proposed action for control.

Class	Definition	Proposed Action for Control
1	Known mutagenic carcinogens	Control at or below compound-specific acceptable limit
2	Known mutagens with unknown carcinogenic potential	Control at or below acceptable limits (appropriate TTC)
3	Alerting structure, unrelated to the structure of the drug substance, no mutagenicity data	Control at or below acceptable limits (appropriate TTC) or conduct bacterial mutagenicity assay If non-mutagenic = Class 5 If mutagenic = Class 2
4	Alerting structure, same alert in the drug substance or compounds related to the drug substance (e.g., process intermediates) which have been tested and are non-mutagenic	Treat as non-mutagenic impurity
5	No structural alerts, or alerting structure with sufficient data to demonstrate lack of mutagenicity or carcinogenicity	Treat as non-mutagenic impurity

Adapted from ICH, 2017.

Table 2
Safety factors described in ICH M7 for the application of LTL methodology to ICH M7 Class 1, 2, and 3 Impurities.

Duration of Treatment	≤1 month	>1–12 months	>1–10 years	>10 years lifetime
Daily intake for Class 2 and 3 (μg/day)	120	20	10	1.5
Daily intake Class 1 (μg/day)	80 x AI	13.3 x AI	6.7 x AI	AI[a]
Safety Factor from Straight Linear Extrapolation	10-300x	5-60x	1-10x	1-7x

[a] Compound-Specific AI.

There are increasing safety factors applied to AIs for short-duration exposures i.e., for the lowest durational periods, the safety factors are 10–300 for ≤1 month and 5–60 for >1–12 months. Less than 6 months, AI determination is based on a probability of 1 in 1 million excess risk of cancer.

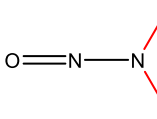
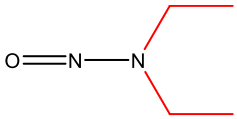
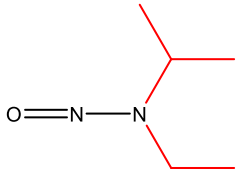
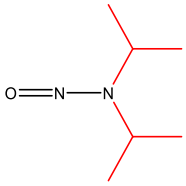
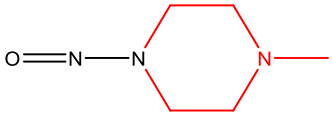
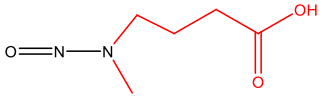
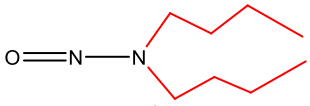
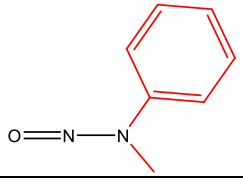
N-Nitrosamines have been discovered in certain medicinal products, including sartans containing a tetrazole ring, pioglitazone, ranitidine, nizatidine, and the biguanide metformin (USFDA, 2020b). *N*-Nitrosamines as a class of impurities are considered cohort-of-concern (COC) compounds as some members of this class are highly potent carcinogens in experimental animals (Kroes et al., 2004). As a result, Health Authorities have requested *N*-nitrosamine risk assessments for all commercial medicinal products (EMA, 2020b; Health Canada, 2020; Swissmedic, 2020; USFDA, 2020a). In the EMA Questions and Answers document and Assessment Report (Procedure under Article 5(3)), AIs were provided for common *N*-nitroso compounds (Table 3) (EMA, 2020b). For three compounds, *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), and *N*-nitrosomethylphenylamine, the AIs were developed from compound-specific carcinogenicity data using linear extrapolation from the relevant TD_{50} (dose that represents a 50% increase in tumor incidence over background). AIs for the all other *N*-nitroso compounds currently, and conservatively, are based on the AIs for the highly potent animal carcinogens NDMA and NDEA. In addition, EMA derived a class-specific limit of 18 ng/day applied to *N*-nitrosamines without carcinogenicity data (EMA, 2020a). The class-specific limit can be adjusted based on a structure activity relationship (SAR) analysis and comparison with other *N*-nitrosamines that have established carcinogenicity data.

Recently there have been some concerns expressed over using the LTL framework for *N*-nitrosamines (EMA, 2020a), although a previous EMA Questions-and-Answer document advocated the application of the LTL concept when calculating *N*-nitrosamine limits (EMA, 2020c). The concern is that higher exposures over an LTL duration would result in an unacceptable excess risk of cancer. Therefore, it is considered critical to fully understand the impact of the ICH M7 LTL framework on excess cancer risk for *N*-nitrosamines.

NDEA is a well-studied compound for the quantitative aspects of carcinogenicity, including time-dependence effects. Druckrey discovered a time-dependence between the daily dose of NDEA and carcinogenicity (Druckrey, 1967). When administered in the drinking water to BD II rats, as the daily dosage (in mg/kg) increased, the time to a 50% induction (T_{50}) in tumor development decreased. With higher daily doses, the total lifetime dose that was required for a 50% response in tumor development over background (D_{50}) increased. For example, with daily dosages of 0.075 and 14.2 mg/kg/day, the D_{50}s were 64 and 1,000 mg/kg and the T_{50}s were 840 and 68 days, respectively. As a result, the dose/time equation was revised for NDEA to $C \times T^{2.3} = k$, which was calculated from the empirical relationship of D_{50}s and T_{50}s. Further testing of NDEA with multiple doses administered in the drinking water to Colworth rats supported the revised equation (Peto et al., 1991a). This showed that NDEA carcinogenicity was based on both dose and time, the latter having a greater influence.

Given the numerous carcinogenicity assays performed with NDEA

Table 3
EMA AIs for *N*-Nitrosamines.

N-Nitros- (CAS#)	Structure	AI (ng/day)	Rationale
Dimethylamine (NDMA) (62-75-9)		96	Based on CPDB TD_{50} Harmonic Mean[a]
Diethylamine (NDEA) (55-18-5)		26.5	Based on CPDB TD_{50} Harmonic Mean[a]
Ethylisopropylamine (16339-04-1)		26.5	NDEA AI
Diisopropylamine (601-77-4)		26.5	NDEA AI
1-Methyl-piperazine (16339-07-4)		26.5	NDEA AI
Methyl-3-carboxypropylamine (61445-55-4)		96	NDMA AI
Dibutylamine (924-16-3)		26.5	NDEA AI
Methylphenylamine (614-00-6)		34.3	Based on CPDB TD_{50}[a,b]

Adapted from EMA, 2020b.
AI – Acceptable Intake, CPDB – Carcinogenicity Potency Database.
[a] https://carcdb.lhasalimited.org/carcdb-frontend/.
[b] Based on esophageal tumors in Sprague Dawley rats of mixed sexes following 104 weeks of exposure in the drinking water. The reported harmonic mean TD_{50}s from Lhasa Carcinogenicity Database and CPDB are 106 and 142 μg/kg/day, respectively.

exposure durations, this compound was used as a case study for LTL principles. The goal of this manuscript is to use existing NDEA animal data to determine if applying the ICH M7 LTL framework would control exposures to acceptable excess cancer risks in humans. As such, these analyses may inform whether the use of LTL AIs for *N*-nitrosamines is generally applicable.

2. Materials and methods

2.1. Data collection

A literature search was performed for NDEA carcinogenicity studies in rats and mice at different durations of exposure. There are two important durational variables required for the calculation of a TD_{50}. The first is **experimental time** or the duration animals are on study and then sacrificed to determine the incidence of tumors. The second is **duration of exposure**, which is the time the compound was dosed in the study. Both are expressed as a percentage of lifetime exposure in a rodent bioassay (104 weeks). For example, if animals were dosed for 10 weeks and then sacrificed after 52 weeks, then the duration of exposure and experimental time are 10% (10/104 weeks [2-year bioassay] x 100) and 50% (52/104 weeks x 100), respectively.

Studies selected required a minimum experimental time of at least 25% of lifetime to ensure that there was sufficient time for tumors to arise. Tumor incidence based on organ site was required to be reported in the study, so that a risk estimate could be calculated. The studies selected needed a minimum number of animals per dose group (≥10).

Each species and sex were analyzed separately for tumor incidence. The doses tested, duration of dosing, route of administration, and number of animals per dose group were documented. The most sensitive organ site was identified for each species and sex. The total tumor incidence for each organ site (totaling all lesions including adenomas and carcinomas) was collected for analysis. The percent of lifetime dosed (experimental dose) was determined from each study by dividing

the dosing duration in weeks by 104. The duration of exposure also factored dosing regimens per week; for example, the duration of exposure was reduced ~30% if compound was administered on only 5 days a week.

2.2. Calculation of duration-specific TD_{50}s

The TD_{50}s were re-calculated according to methodology developed from the carcinogenicity potency database (CPDB) (Gaylor and Gold, 1995; Peto et al., 1984; Sawyer et al., 1984). The difference however is that TD_{50}s will be specific to a duration of exposure whereas the TD_{50}s in the CPDB are corrected to a lifetime value. The TD_{50} is calculated from Equation (1).

$$-\ln\left(-\left[\frac{P-P_0}{1-P_0}-1\right]\right)=\beta\cdot D \qquad \text{Equation 1}$$

Where D is the dose, P is the proportion of animals with the specified tumor type observed at a certain D, and P_0 is the proportion of animals with the specified tumor type for the control. β is the slope used to

Table 4
Summary of NDEA Carcinogenicity Studies used for LTL Analysis.

Species/ Strain/Sex/ Age of animal	Doses (mg/kg/day)	Duration of Dosing	Time of Sacrifice for Necropsy/ Histopathology	Route of Administration	# Animals/ Dose Group	Most Sensitive Organ	$TD_{50\ doe}$ (μg/kg)	% of lifetime dosed	Reference
Rat/ Colworth/ M/6 wks	0, 0.001, 0.003, 0.005, 0.01, 0.02, 0.041, 0.061, 0.082, 0.102, 0.122, 0.163, 0.204, 0.245, 0.326, 0.653	Lifetime	Lifespan	Drinking Water	C – 240 T - 60	Liver	265	100%	Peto et al. (1991b)
Rat/ Colworth/ F/6 wks	0, 0.002, 0.004, 0.009, 0.018, 0.036, 0.072, 0.107, 0.143, 0.179, 0.215, 0.287, 0.358, 0.430, 0.573, 1.146	Lifetime	Lifespan	Drinking Water	C - 240 T - 60	Liver	226	100%	Peto et al. (1991b)
Rat/Sprague-Dawley/M/ 14 wks	0, 0.01, 0.032, 0.1	Lifetime (5x per wk)	Lifespan	Drinking Water	C – 500 T - 80	Liver	128	71%	Berger et al. (1987)
Rat/Fischer/ F/6–8 wks	0, 0.026[a]	Lifetime (5x per wk)	Lifespan	Drinking Water	C – 20 T - 20	Esophagus	30	71%	Lijinsky et al. (1981)
Rat/Sprague-Dawley/M/ NA	0, 0.1	Lifetime (5x per wk)	Lifespan	Drinking Water	C- 82 T - 80	Liver	116	71%	Habs and Schmahl (1980)
Rat/Wistar-OSU/F/W	0, 0.2	60 wks	60 wks	Drinking Water	C – 18 T - 20	Liver	200 (67[b])	58%	Nixon et al. (1974)
Rat/Wister-OSU/M/W	0, 0.2	60 wks	60 wks	Drinking Water	C – 17 T-18	Liver	552 (184[b])	58%	Nixon et al. (1974)
Rat Fischer/ F/6–8 wks	0, 0.026, 0.063[a]	60 wks (5x per wk)	Lifespan	Drinking Water	C – 20 T - 20	Liver	165	41%	Lijinsky et al. (1981)
Rat/Wistar-OSU/F/W	0, 1	30 wks	30 wks	Drinking Water	C – 18 T – 20	Liver	660 (56[b])	29%	Nixon et al. (1974)
Rat Wistar-OSU/M/W	0, 1	30 wks	30 wks	Drinking Water	C – 17 T - 19	Liver	519 (43[b])	29%	Nixon et al. (1974)
Rat/F344/F/ 7–8 wks	0, 0.4[a]	30 wks (5x per wk)	Lifespan	Drinking Water	C – 20 T - 20	Esophagus	172	21%	Lijinsky et al. (1983)
Rat/Fischer/ F/6–8 wks	0, 0.026, 0.063, 0.16[a,c]	30 wks (5x per wk)	Lifespan	Drinking Water	C – 20 T – 19-20	Liver	310	21%	Lijinsky et al. (1981)
Rat/Fisher/F/ 6–8 wks	0, 2.57[a]	22 wks (5x per wk)	Lifespan	Drinking Water	C – 20 T - 20	Liver	2,960	15%	Lijinsky et al. (1981)
Rat/Fisher/F/ 6–8 wks	0, 6.46[a]	17 wks (5x per wk)	Lifespan	Drinking Water	C – 20 T - 20	Liver	2,360	12%	Lijinsky et al. (1981)
Rat/Sprague-Dawley/M/ 12 wks	0, 1.25, 2.5, 5, 10, 20, 40, 80, 160	Single Dose	Lifespan	Intravenous	C – 10 T – 10	Kidney	226,950	0.1%	Mohr and Hilfrich (1972)
Rat/Sprague-Dawley/F/ 12 wks	0, 1.25, 2.5, 5, 10, 20, 40, 80, 160	Single Dose	Lifespan	Intravenous	C – 10 T - 10	Kidney	67,835	0.1%	Mohr and Hilfrich (1972)
Rat F344/M/ 5 wks	75	Single Dose	79 wks	Intraperitoneal	T - 19	Liver	113,104[d] (65,263[b])	0.1%	Diwan et al. (2001)
Mouse/ C57BL/ 6NCr/M/5 wks	0, 90	Single Dose	47 wks	Intraperitoneal	C – 27 T - 28	Liver	404,604 (82,634[b])	0.1%	Beebe et al. (1995)
Mouse/ B6D2F1/ M/5 wks	0, 90	Single Dose	47 wks	Intraperitoneal	C – 34 T - 33	Liver	261,607 (53,429[b])	0.1%	Beebe et al. (1995)
Mouse/DBA/ 2NCr/M/5 wks	0, 90	Single Dose	47 wks	Intraperitoneal	C – 23 T - 28	Liver	258,623 (52,820[b])	0.1%	Beebe et al. (1995)

C- Control, T – Treated, wk – week, NA – Not Available, W - Weanling.
[a] Converted from mg/L to mg/kg/day based on CPDB assumptions.
[b] $TD_{50\ doe,lc}$ – converted because experiment time ended prior to a lifetime.
[c] Doses where total tumor incidence per organ site was reported.
[d] No controls reported in the study. $TD_{50\ doe}$ calculated assuming zero tumor incidence with same number of animals tested as treated.

calculate the $TD_{50\ doe}$ (TD_{50} based on duration of exposure) as shown in Equation (2).

$$TD_{50\ doe} = \frac{0.693}{\beta} \quad \text{Equation 2}$$

Conversions of dose from levels in the drinking water were developed using CPDB assumptions, including standard lifespan, water consumption (mL/day) and body weight (https://files.toxplanet.com/cpdb/methods.html#estimation) unless otherwise reported in the study. While the duration of exposure was not corrected, the experimental time was corrected if the study was terminated prior to the animals' lifetime. This is because even if duration of exposure is limited, it is still important to estimate if tumors will develop following cessation of treatment. However, when time of sacrifice was prior to a lifetime, the TD_{50} was corrected (Equation (3) – $TD_{50\ doe,lc}$ – duration of exposure, lifespan corrected) based on experimental time (ExpTime) to adjust for tumor development over a lifetime in accordance with CPDB methods. In Diwan (2001) no control was tested, and so it was conservatively assumed that background incidence is zero.

$$TD_{50\ doe,\ lc} = TD_{50\ doe} \cdot \left(\frac{ExpTime}{104\ weeks}\right)^2 \quad \text{Equation 3}$$

3. Results

The studies collected from the literature search for NDEA carcinogenicity data are listed in Table 4. The rat and mouse dose-response data included chronic and short-term exposure durations with many different strains tested. The details of each investigation were divided into species/sex from each study with a total of 20 different $TD_{50\ doe}$ values calculated, and 8/20 (40%) converted to $TD_{50\ doe,lc}$ since the experiment was terminated prior to 104 weeks. Most data were from drinking water-studies (14/20–70%), while the rest (6/20–30%) were parenteral (intraperitoneal or intravenous) studies. The organ most sensitive to the carcinogenic effects of NDEA among the studies considered was the liver (16/20–80%). There was a wide range of durations for the different animal exposures (0.1%–100% of a lifespan).

From the studies listed in Table 4, Peto et al. (1991b) was considered the most robust, testing 15 concentrations with a total of more than 2000 animals. However, several studies used to calculate the TD_{50}s in the LTL approach had lower data quality than a typical bioassay used to derive an AI, for example, less than 50 animals/sex and less than 3 dose levels (Thresher et al., 2019). Studies by Beebe et al. (1995) and Diwan et al. (2001) were conducted using only one sex and carcinogenicity was assessed after a single dose with the number of treated animals ranging from 19 to 33. Mohr and Hilfrich (1972) had the most limited number of treated animals (10) and reported kidney tumors, whereas the liver and esophagus are typically the most sensitive organ sites for NDEA.

An LTL NDEA analysis based on the ICH M7 framework was compared to data from empirical carcinogenicity studies (Table 5). The exposure durations were divided to match the durations in ICH M7 used for LTL AIs with Class 1, 2 and 3 impurities (Table 2). Also, the durations are reported based on estimated percent of lifetime, assuming a human lifetime of 70 years. The lifetime AI for NDEA of 26.5 ng/day adopted by regulatory agencies is based on the CPDB harmonic-mean TD_{50} (EMA, 2020a; EMA, 2020b; Health Canada, 2020; Swissmedic, 2020; USFDA, 2020a). The LTL AI calculations for NDEA are based on those set out in the ICH M7 guideline. The animal duration-of-exposure percentages (relative to lifetime) were split into three categories (≤1%, >1–15%, and >15–100%), instead of four because no studies were found within the narrow range >0.1–1.0%. The most datapoints (n = 12) were derived from studies with chronic exposures (>15–100% of a lifetime), and the AI from the lowest calculated $TD_{50\ doe,lc}$ was 30 ng/day, which is consistent with the AI of 26.5 ng/day mentioned above. Two datapoints were identified that were derived from studies that correspond to >1–15% of a lifetime. In this category, the NDEA AI calculated from the lowest $TD_{50\ doe,lc}$ (2,360 ng/day) was 13.2x greater than the AI for >1–15% of a lifetime using ICH M7 LTL methodology (178 ng/day). The NDEA AIs calculated using ICH M7 LTL methodology for ≤1% of a lifetime, ranged from 3,52.5 to 2,120 ng/day. The lowest AI estimated from the empirical $TD_{50\ doe}$ values is 52,820 ng/day, which is 25 - 150-fold greater than the NDEA LTL AIs derived using ICH M7 methodology.

Table 5
LTL analysis for NDEA for rats and mice based on an empirical analysis of the literature.

Duration of exposure (ICH M7)	≤1 month	>1 month–1 year	>1 year–10 years	>10 years – lifetime[a]
% of lifetime based on ICM M7 duration cutoffs	≤0.1%	>0.1–1%	>1–15%	>15%–100%
AI based on duration of exposure (ng)	2,120	352.5	177.6	26.5
TD_{50} based on duration of exposure (μg/kg)	2,120	352.5	177.6	26.5
Duration Ranges of Animal Studies	≤1%		>1–15%	>15–100%
Empirical $TD_{50\ doe}$ values based on duration of exposure (μg/kg) (number of different animal groups)[a,b]	52,820–226,950 (n = 6)		2,360–2,960 (n = 2)	30–310 (n = 12)
Lowest AI calculated based on empirical $TD_{50\ doe}$ values (ng/day)	52,820		2,360	30
Margin of Safety Lowest Empirical TD_{50}/AI	24.9	149.8	13.2	1.1

[a] Assuming a lifetime of approximately 70 years.
[b] Adjusted for experimental time if terminated prior to 104 weeks ($TD_{50\ doe,lc}$).

4. Discussion

The analysis herein confirms that the LTL principles described in ICH M7(R1) would control *N*-nitrosamine impurity exposure to a negligible excess risk of cancer by using a case study of the well-studied compound, NDEA. NDEA has been used as a reference compound to generate AIs for 5 out of the 8 *N*-nitrosamines for which limits have been recommended by EMA (EMA, 2020b). In general, for the highly-potent small-molecule, alkyl-amine *N*-nitrosamines, the mechanism of action for mutagenicity is very similar, i.e., α-hydroxylation leading to diazonium-ion formation, and resulting in alkylation of DNA bases (Guttenplan, 1987a; Lijinsky, 1987a). In addition, a similar dose-time relationship has been shown for 65 other *N*-nitrosamines (Druckrey, 1967; Druckrey et al., 1967; Peto et al., 1991a). For other types of *N*-nitrosamines there can be different types of mechanisms for mutagenicity and carcinogenicity, depending on various chemical factors such as steric hindrance at the alpha-carbon, chain length, and polarity (Guttenplan, 1987b; Helguera et al., 2007, 2008, 2010; Lijinsky, 1987b). Steric hindrance at the alpha-carbon can reduce mutagenic potential and carcinogenic potency. Longer-chain length *N*-nitrosamines can result in metabolism at the β- or ω-carbon. Increasing polarity can facilitate excretion before the site of metabolism at the liver or result in metabolism outside the liver. The result is that while small-chain alkyl-nitrosamines tend to be more sensitive for liver/esophagus tumors in animals, other *N*-nitrosamines are more sensitive in the bladder (*N*-nitrosamines with polar substituents), or nasal cavity (heterocyclic *N*-nitrosamines) (Buist et al., 2015). Nonetheless, this report shows that for a COC *N*-nitrosamine, NDEA, the framework established by Felter et al. (2011) and ICH M7 for LTL exposures to carcinogens is conservative for controlling to a negligible excess cancer risk.

Concern has been raised that the LTL approach "relies on strict linearity of the dose-response even in the higher dose ranges which is unproven" and acutely overwhelming the repair capacity of human DNA (EMA, 2020a). Low dose, linear extrapolation from the TD_{50} assumes that there is no DNA repair and threshold for carcinogenicity, resulting in an AI that is well below biological responses that would prevent a small increased incidence of cancer within a large human population. It is important to understand if cancer risk in the population would be increased when comparing high-dose LTL versus low dose chronic exposures. A series of LTL stop-exposure animal studies have been performed by the National Toxicology Program (NTP), which compared carcinogenic potencies in high-dose short-term exposures with those from chronic studies (Halmes et al., 2000). Stop-exposure studies follow the same general protocol as a 2-year bioassay, but the animals are exposed over a limited duration at higher doses. For each tumor response observed in a bioassay, an ED_{01} was calculated (dose yielding an excess cancer risk of 1% over background for a specific tumor type). The results suggested that differences in carcinogenic potency (ED_{01}) from chronic to LTL exposures varied within an order of magnitude, which is rather small given the variability of response for a bioassay. In addition, dose-rate correction factors are applied for the extremely short LTL exposures to ensure safety over these short durations (Bos et al., 2004; Felter et al., 2011).

Note 6 of the ICH M7 guideline compared LTL limits based on a strict linear relationship of a theoretical cancer risk during short-term exposures and the actual proposed LTL AIs. ICH M7(R1) states "These proposed levels are in general significantly lower than the calculated values thus providing safety factors that increase with shorter treatment durations." For durations less than 6 months, the excess cancer risk from LTL AIs generated in ICH M7 are lower at a 1 in 1 million excess cancer risk rather than a 1 in 100,000 excess cancer risk for lifetime exposure. Therefore, LTL AIs for *N*-nitrosamines using existing ICH M7 guidelines would be of negligible excess cancer risk following high exposures over a more limited exposure duration.

The challenge with the ICH M7 LTL framework is that it requires assumptions to extrapolate a tumor response in a human population, which complicates the actual precision of risk. It ignores factors of DNA-repair or the multi-stage process of carcinogenicity which tends to overestimate risk. This study focused on rodents (rats and mice) as the primary species, while non-rodent primate studies were considered too limited in terms of reported details of the study (including length of exposure time), no controls were reported in some cases, mixed species were tested, and no comparator short-term data was available (Adamson and Sieber, 1983; Thorgeirsson et al., 1994).

Mechanisms of DNA repair have been proposed but further knowledge may allow for understanding of differences between humans and tested species. Two major adducts of NDEA are O^6 guanine (O^6-ethylguanine) and O^4 thymine (O^4-ethylthymine) sites (Verna et al., 1996). O^6-Ethylguanine is efficiently repaired by O^6-alkylguanine-DNA alkyltransferase (AGT). AGT is a widely distributed DNA repair protein which removes alkyl groups to a cysteine residue of the enzyme (Pegg, 2011). AGT is a suicide enzyme in that it can become saturated for DNA repair, until new enzymes can be produced. AGT prefers repair of O^6-ethylguanine, but it can also repair O^4-ethylthymine (Fang et al., 2010). O^4-Ethylthymine is repaired much more slowly (up to 19 days in rat liver) than O^6-ethylguanine (Verna et al., 1996). Thus, O^4-ethylthymine adduct will accumulate at a faster rate over time given the slow repair. Humans contain a significantly greater amount of AGT activity per μg DNA than both rat and mouse in liver and other tissues (Gerson et al., 1986). However, AGT enzymes are induced in rat liver cells following response to a supraphysiological dose of a DNA-damaging agent (Fritz and Kaina, 1992; Fritz et al., 1991). Overall species differences between humans and rodents for repair of adducts due to NDEA is unknown.

Interspecies extrapolation of tumor development is difficult to translate to humans, and it is also difficult to understand causation in a large human population. Epidemiology studies are limited by the number of patients analyzed and the length of follow-up time. Environmental exposures are variable, whereas animal exposures can be maintained to a controlled, constant amount. Laboratory animals cannot replicate the diversity of patients, especially since patients can be compromised by disease. Humans also have background exposures to *N*-nitrosamines from the air, food, water, and tobacco products, and are exposed endogenously as well, which can be controlled with laboratory animals (Fristachi and Rice, 2007; Gushgari and Halden, 2018; Hrudey et al., 2013; Krul et al., 2004; Lee, 2019; Snodin and Elder, 2019; Zeilmaker et al., 2010). As a result, epidemiology studies have observed mixed results regarding association of *N*-nitrosamine impurities in pharmaceuticals and cancer (Fukushima et al., 2010; Iwagami et al., 2020; Kantor et al., 2020; McGwin, 2020; Pottegard et al., 2018; Yoon et al., 2021; Zeng and Mitch, 2016). These limitations caution the interpretation of cancer risk estimation, yet the study supports that the ICH M7 framework for LTL exposures are conservative even for *N*-nitrosamines.

While *N*-nitrosamines are considered part of the COC class of compounds, there is no evidence to suggest that they would respond differently than any other mutagenic carcinogen in terms of LTL exposure. A single high-dose NDEA animal exposure has been shown to result in a carcinogenic response later in the animal's life (Beebe et al., 1995; Mohr and Hilfrich, 1972; Nixon et al., 1974); however, this is also true of many other carcinogens, with about 426 chemical agents from a wide variety of chemical classes known to cause tumor development from a single high-dose animal exposure (Calabrese and Blain, 1999). The dose required to cause tumors in a single dose study is significantly higher than for chronic exposure, even for a COC like NDEA. For example, TD_{50}s from daily exposure over 100% of a lifespan were 226–265 μg/kg (Table 4). In comparison, the TD_{50}s from a single exposure were 52, 820–226,950 μg/kg when correcting for experimental time.

A comparison of the Druckrey (1967) model ($C \times T^{2.3} = k$) was made with $TD_{50\ doe,lc}$ values and ICH M7 LTL AIs (Fig. 1). The doses generated for each duration were calculated to reflect a 1 in 100,000 excess risk of cancer for a 50 kg person for different durational periods. The Druckrey (1967) model resulted in higher estimated LTL doses for a 1 in 100,000 excess of cancer than both the ICH M7 LTL AIs and the lower of the calculated $TD_{50\ doe,lc}$ values for the extremely short durations of exposure ($\leq$1% of a lifetime). The difference between values derived from Druckrey (1967) and $TD_{50\ doe,lc}$ is most likely because studies undertaken by Druckrey employed a single species tested and testing laboratory, and thus exhibited a less-variable response. Studies gathered to derive $TD_{50\ doe,lc}$ values involve different study designs, laboratory environments, and strains of animals. Therefore, this paper reflects a conservative estimate of the cancer risk over short-term exposure while the model developed by Druckrey (1967) may reflect a more accurate estimate of dose versus time for NDEA carcinogenicity for a specific species/strain. More importantly, AIs developed using the ICH M7 framework would result in cancer risk estimates that would be below a 1 in 100,000 or 1 in 1 million (for LTL exposures $\leq$1% of a lifetime) when comparing to the Druckrey (1967) model or based on empirical data gathered for the purposes of this publication.

5. Conclusions

The LTL framework included in ICH M7 for Class 1–3 pharmaceutical impurities is of critical importance to derive appropriate AIs that are specific to the duration of a licensed treatment or for a clinical trial. The LTL AIs were designed to be conservative, with safety factors increasing for shorter exposures. Empirical carcinogenicity data from different NDEA exposure durations indicate that the cancer risk from the ICH M7 derived LTL AIs would be below a 1 in 100,000 excess cancer risk and below a 1 in 1 million excess cancer risk for extremely short (<6 months) durations. For NDEA, the LTL AIs that follow the ICH M7 framework and would be protective from a patient safety perspective are listed in Table 6. *N*-Nitrosamines, despite having the potential to be potent

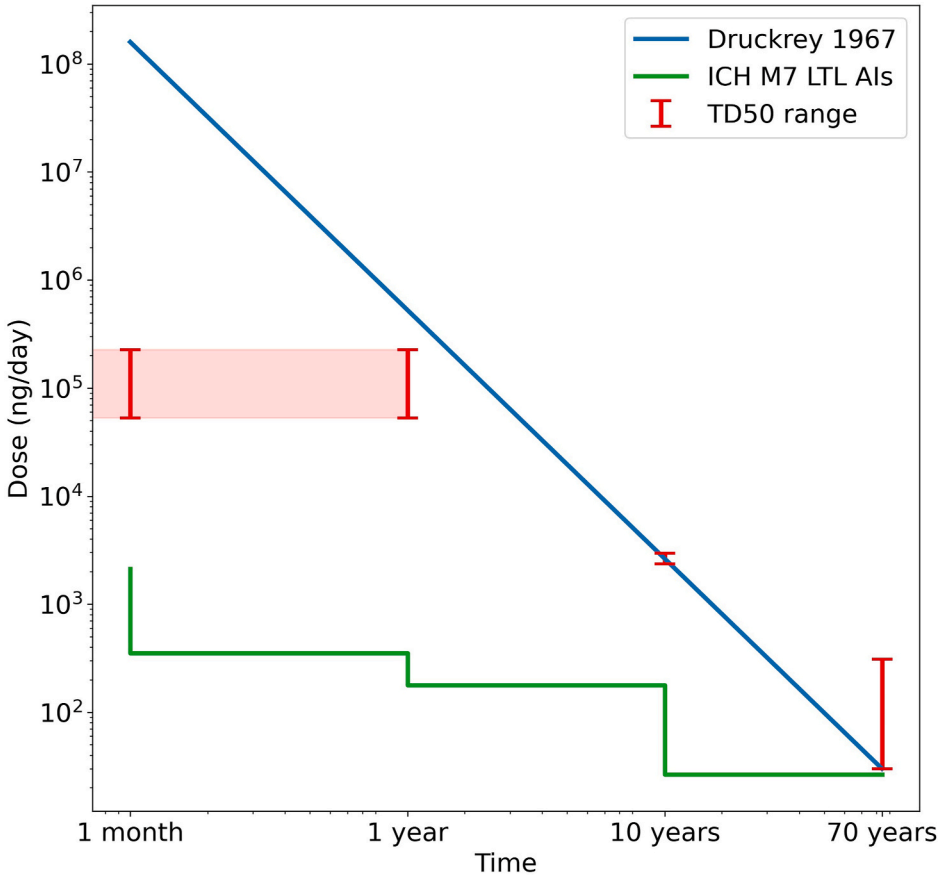


Fig. 1. Relationship between the tumor model predicted by Druckrey (1967) (i.e., C x $T^{2.3}$), empirical data used to develop $TD_{50\ doe,lc}$ (referred to as TD_{50} in the figure) values and ICH M7 LTL AIs (log scales represented). Doses converted to ng/day assuming a 50 kg person. The values represent doses that are considered ≤1 in 100,000 excess risk of cancer. The ICH M7 LTL AIs which are less than 6 months in duration are also ≤1 in 1 million excess risk of cancer. The red shaded region indicates that durations of ≤1% of a lifetime were combined. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Proposed NDEA LTL limits per ICH M7 principles.

Duration of<!–Soft-enter Run-on– > treatment	≤1 month	1–12 months	≤10 year	More than 10 years
Total daily intake (μg/day)	2.1	0.352	0.178	0.0265

mutagenic animal carcinogens, should be controlled using the same ICH M7 framework for LTL exposures applied to other classes of compounds which are potential mutagenic carcinogens.

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Declaration of competing interest

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